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SELECTIVE ENZYME PURIFICATION BY AFFINITY CHROMATOGRAPHY

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The purification of proteins by conventional procedures is frequently laborious and incomplete, and the yields are often low. Enzyme isolation based on a highly specific biological property—strong reversible association with specific substrates or inhibitors—has received only limited attention.[1–4]

In affinity chromatography, the enzyme to be purified is passed through a column containing a cross-linked polymer or gel to which a specific competitive inhibitor of the enzyme has been covalently attached. All proteins without substantial affinity for the bound inhibitor will pass directly through the column, whereas one that recognizes the inhibitor will be retarded in proportion to its affinity constant. Elution of the bound enzyme is readily achieved by changing such parameters as salt concentration or pH, or by addition of a competitive inhibitor in solution.

The successful application of the method requires that the adsorbent have a number of favorable characteristics. Thus, the unsubstituted matrix or gel should show minimal interaction with proteins in general, both before and after coupling to the specific binding group. It must form a loose, porous network that permits easy entry and exit of macromolecules and which retains favorable flow properties during use. The chemical structure of the supporting material must permit the convenient and extensive attachment of the specific ligand under relatively mild conditions, and through chemical bonds that are stable to the conditions of adsorption and elution. Finally, the inhibitor groups critical in the interaction must be sufficiently distant from the solid matrix to minimize steric interference with the binding processes.

In this report the general principles and potential application of affinity chromatography are illustrated by results of its application to the purification of staphylococcal nuclease, α-chymotrypsin, and carboxypeptidase A. The solid matrix used in these studies was Sepharose (a "beaded" form of the cross-linked dextran of highly porous structure, agarose[5]) which displays virtually all the desirable features listed above. Activation of the Sepharose by treatment with cyanogen bromide[6, 7] results in a derivative that can be readily coupled to unprotonated amino groups of an inhibitory analog. The resultant Sepharose-inhibitor gel is a highly stable structure which has nearly ideal properties for selective column chromatography.

Experimental Procedure.—Materials: Sepharose 4B was obtained from Pharmacia, cyanogen bromide from Eastman, pdTp and benzoyl-L-tyrosine ethyl ester from Calbiochem.

Staphylococcal nuclease (Foggi strain) was obtained by modification[8] of techniques described by Fuchs *et al.*[9] The following purified enzymes were purchased from Worthington: α-chymotrypsin (CDS 7LC); α-chymotrypsin, DFP-treated (CD-DIP 204); chymotrypsinogen A (CGC 8CC); subtilisin VIII (66B 3080); trypsin, DFP-treated

(TDIP 7HA); pancreatic ribonuclease A (RAF 8BA); carboxypeptidase A (COA DFP 8FB); carboxypeptidase B (COB DFP 7GA). Beef pancreas acetone powder (26B-8890) was obtained from Sigma.

3′-(4-Amino-phenylphosphoryl)-deoxythymidine - 5′ - phosphate (Fig. 1) was synthesized from pdTp.[10] The following compounds were prepared by classical methods of peptide synthesis: L-tyrosyl-D-tryptophan, D-tryptophan methyl ester, ϵ-amino caproyl-D-tryptophan methyl ester, and β-phenylpropionamide.

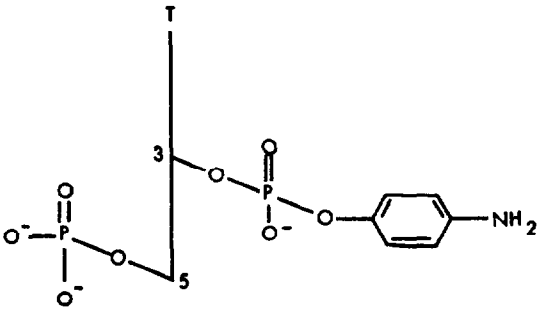

FIG. 1.—Structure of nuclease inhibitor used for attachment to Sepharose.

Preparation of substituted Sepharoses: Cyanogen bromide activation of Sepharose was based on procedures previously described.[6, 7] Sepharose (decanted) is mixed with an equal volume of water, and cyanogen bromide (100 mg per ml of settled Sepharose) is added in an equal volume of water. The pH is immediately adjusted to, and maintained at, 11 by titration with 4 *N* NaOH. When the reaction has ended (about 8 min), the Sepharose is washed with about 20 vol of cold 0.1 *M* $NaHCO_3$ on a Buchner funnel under suction (about 2 min). The washed Sepharose is suspended in cold 0.1 *M* $NaHCO_3$, pH 9.0, or another appropriate buffer, in a volume equal to that of the original Sepharose, and the inhibitor is quickly added in a solution representing 5–15% of the final volume. This mixture is stirred gently at 4° for 24 hr, after which it is washed extensively with water and buffer.

The quantity of inhibitor coupled to the Sepharose can be controlled by the amount of inhibitor added to the activated Sepharose, or by adding very large amounts of inhibitor to yield maximal coupling, followed by dilution of the final Sepharose-inhibitor with unsubstituted Sepharose. Furthermore, to increase the amount of inhibitor coupled to the Sepharose, it is possible to repeat the activation and coupling procedures on the already substituted material, provided the inhibitor is stable at pH 11 (for 10 min). In the cases reported here the amount of inhibitor that was coupled was easily estimated by calculating the amount of inhibitor (spectroscopically measured) which was not recovered in the final washings. Alternatively, acid hydrolysis of the Sepharose, followed by amino acid analysis, could be used for quantitation in those cases where amino acids or peptides are coupled to the Sepharose.

The operational capacity of the Sepharose columns for adsorption of protein was determined by two methods. In the first, an amount of enzyme in excess of the theoretical capacity was added to a small column (about 1 ml). After washing this column with buffer until negligible quantities of protein emerged in the effluent, the enzyme was rapidly removed (i.e., by acetic acid washing) and its amount determined. In the second method, small aliquots of pure protein were added successively to the column until significant protein or enzymatic activity emerged; the total amount added, or that which was subsequently eluted, was considered the operational capacity.

Results.—Staphylococcal nuclease: This extracellular enzyme of *Staphylococcus aureus*, which is capable of hydrolyzing DNA and RNA, is inhibited, competitively, by pdTp (see ref. 11 for recent review). Thymidine 3′,5′-di-*p*-nitrophenylphosphate is a substrate for this enzyme, which rapidly releases *p*-nitrophenylphosphate from the 5′-position and, slowly, *p*-nitrophenol from the 3′-position.[12] The presence of a free 5′-phosphate group, however, endows various synthetic derivatives with strong inhibitory properties.[12] 3′-(4-Amino-phenyl-

phosphoryl)-deoxythymidine-5′-phosphate[10] (Fig. 1) was selected as an ideal derivative for coupling to Sepharose for affinity chromatography, since it has strong affinity for nuclease (K_i, 10^{-6} M), its 3′-phosphodiester bond is not cleaved by the enzyme, it is stable at pH values of 5–10, the pK of the amino group is low, and the amino group is relatively distant from the basic structural unit (pTp-X) recognized by the enzymatic binding site.[12]

This inhibitor could be coupled to Sepharose with high efficiency, and the resulting inhibitor-Sepharose shows a high capacity for nuclease (Table 1). Columns containing this substituted Sepharose completely and strongly adsorbed samples of pure or partially purified nuclease (Fig. 2). If the amount of nuclease applied to such columns does not exceed half of the operational capacity of the Sepharose, virtually no enzyme activity escapes in the effluent, even after washing with a quantity of buffer more than 50 times the bed volume of the column. Elution of nuclease could be effected by washing with buffers having a pH inadequate for binding (less than 6). The yields of protein and activity were invariably greater than 90 per cent. Acetic acid (pH 3) was a convenient eluant since, with this solvent, the protein emerged sharply (in a few tubes) and the material could be directly lyophilized. The purity of the nuclease obtained in these studies was confirmed by its specific activity, immunodiffusion,[9] and disc gel electrophoresis.[9]

Such columns can be used for rapid and effective large-scale purification of nuclease. For example, 110 mg of pure nuclease could be obtained from a crude nuclease concentrate (the same sample used in Fig. 2) with a 20-ml Sepharose column in an experiment completed in 1.5 hours. When the total concentration of protein in the sample exceeded 20–30 mg per ml, small amounts of nuclease appeared in the first peak of protein impurities, especially if very fast flow rates were used (400 ml/hr). However, with such flow rates, nuclease could still be completely extracted if more dilute samples were applied. The columns used in these experiments could be used repeatedly, and over protracted periods, without detectable loss of effectiveness.

Nuclease treated with cyanogen bromide, which is enzymatically inactive, was not adsorbed or retarded by the nuclease-specific Sepharose columns. Spleen phosphodiesterase, which, like staphylococcal nuclease, hydrolyzes DNA and RNA to yield 3′-phosphoryl derivatives but which is not inhibited by the 5′-phosphoryl nucleotides, passed unretarded through these columns.

A dramatic illustration of the value of these techniques in enzyme purification

TABLE 1. *Efficiency of coupling of 3′-(4-amino-phenylphosphoryl)-deoxythymidine-5′-phosphate (Fig. 1) to Sepharose, and capacity of the resulting adsorbent for staphylococcal nuclease.*

Expt.	μMoles of Inhibitor/ml Sepharose Added	μMoles of Inhibitor/ml Sepharose Coupled*	Mg of Nuclease/ml Sepharose Theoretical†	Mg of Nuclease/ml Sepharose Found
A	4.1	2.3	44	...
B	2.5	1.5	28	8
C	1.5	1.0	19	...
D	0.5	0.3	6	1.2

* Determined by the procedures described in the text.
† Assuming equimolar binding.

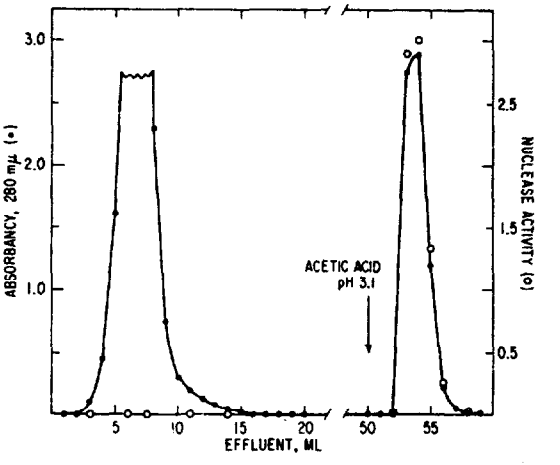

FIG. 2.—Purification of staphylococcal nuclease by affinity adsorption chromatography on a nuclease-specific Sepharose column (0.8 × 5 cm)(sample B, Table 1). The column was equilibrated with 0.05 *M* borate buffer, pH 8.0, containing 0.01 *M* $CaCl_2$. Approximately 40 mg of partially purified material (containing about 8 mg of nuclease) was applied in 3.2 ml of the same buffer. After 50 ml of buffer had passed through the column, 0.1 *M* acetic acid was added to elute nuclease. DNase activity is expressed as the change in absorbancy at 260 mμ caused by 1 μl of sample.[13] 8.2 mg of pure nuclease and all of the original activity was recovered. The flow rate was about 70 ml per hour.

was afforded by the one-step purification obtained by passing a crude culture of *Staphylococcus aureus* through such a nuclease-specific Sepharose column after removal of cells by centrifugation.[14] After adjustment of 500 ml to pH 8, addition of 50 ml 1 *M* $CaCl_2$ was then added to ensure a calcium ion concentration consistent with complex formation. The medium, containing about 6 μg of nuclease per ml, was passed through a 1-ml column with nearly complete adsorption of nuclease activity, which was subsequently eluted with acetic acid.

Affinity columns can also be of use in the separation of active and inactive nuclease derivatives, as from samples alkylated with iodoacetic acid[15] or subjected to proteolytic digestion.[16] The specific Sepharose adsorbent can also be used effectively as an insoluble inhibitor to stop enzymatic reactions (Fig. 3).

α-Chymotrypsin: A number of proteolytic enzymes, of which α-chymotrypsin and carboxypeptidase A are examples, are capable of binding, but not hydrolyzing, significantly, the enantiomeric substrate analog. Therefore, the techniques described here should be applicable to a large number of enzymes of this class.

D-tryptophan methyl ester was the inhibitor coupled to Sepharose in experiments with α-chymotrypsin (Fig. 4). When this inhibitor is coupled directly to Sepharose, incomplete and unsatisfactory resolution of the enzyme results (Fig. 4*B*). However, dramatically stronger adsorption of enzyme occurs if a 6-carbon chain (ε-amino caproic acid) is interposed between the Sepharose matrix and the inhibitor (Fig. 4*C*). This illustrates the marked steric interference that results when the inhibitor is attached too closely to the supporting gel. The importance of specific affinity for the enzyme binding site is illustrated by the absence of adsorption of DFP-treated α-chymotrypsin (Fig. 4*D*). Impurities in commercial α-chymotrypsin constituting 4–12 per cent, could be detected in different lots by these techniques. Greater than 90 per cent of the activity and protein added to these affinity columns could be recovered, and the columns could be used repeatedly without detectable loss of effectiveness.

It is notable that significant retention was obtained with an affinity adsorbent

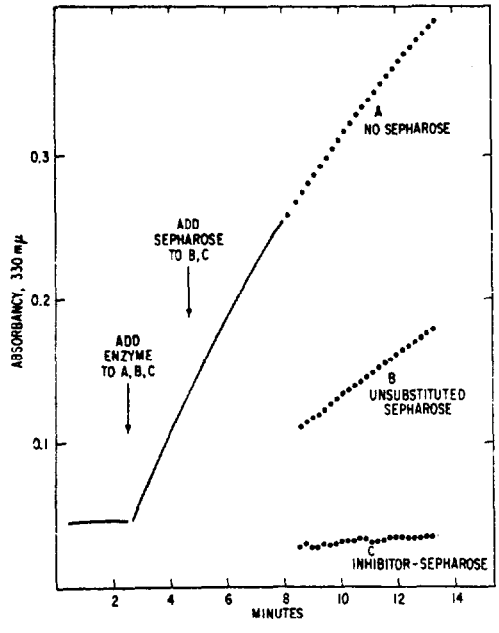

FIG. 3.—Stopping of nuclease-catalyzed reaction by addition of Sepharose inhibitor (sample *A*, Table 1). Ten μg of nuclease was added to each of three samples containing 1.5 ml of 0.05 *M* Tris buffer, pH 8.8, 0.01 *M* $CaCl_2$, and 0.1 mM synthetic substrate, thymidine 3′-5′-di-*p*-nitrophenylphosphate.[10, 12] The change in absorbancy at 330 mμ, representing release of *p*-nitrophenylphosphate, was recorded continuously in cuvette *A*. At 4.6 min, 0.5 ml of untreated Sepharose or of Sepharose coupled with inhibitor were added to *B* and *C*, respectively. After a 2-min centrifugation to remove the Sepharose, the changes in absorbancy were recorded (8 min). The difference in activity between *A* and *B* is due to dilution.

that contained a relatively weak inhibitor, N-ε-amino caproyl-D-tryptophan methyl ester. N-acyl-D-tryptophan esters have K_i values of about 0.1 mM,[17] some 100 times greater than that of the inhibitor used for purification of staphylococcal nuclease. These results suggest that unusually strong affinity constants will not be an essential requirement for utilization of these techniques.

The relatively unfavorable affinity constant of α-chymotrypsin for the D-tryptophan methyl ester derivatives was compensated for by coupling a very large amount of inhibitor to the Sepharose. About 65 μmoles of the compound were present per milliliter of Sepharose during the coupling procedure, and there was an uptake of 10 μmoles per ml, with a resultant effective concentration of inhibitor, in the column, of about 10 mM. Such a high degree of substitution

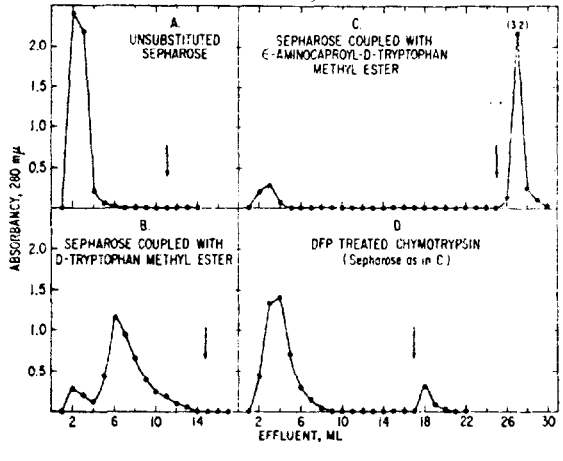

FIG. 4.—Affinity chromatography of α-chymotrypsin on inhibitor Sepharose columns. The columns (0.5 × 5 cm) were equilibrated and run with 0.05 *M* Tris-Cl buffer, pH 8.0, and each sample (2.5 mg) was applied in 0.5 ml of the same buffer. One-milliliter fractions were collected, the flow rate was about 40 ml per hour, and the experiments were performed at room temperature. α-Chymotrypsin was eluted with 0.1 *M* acetic acid, pH 3.0 (*arrows*). Peaks preceding the arrows in *B*, *C*, *D* were devoid of enzyme activity.[18]

occurred despite the relatively high pK of the amino group of the ϵ-amino caproyl derivative. The coupling was done in 0.1 M $NaHCO_3$ buffer, pH 9.0; higher pH values could not be safely used because of the probability of hydrolysis of the ester bond. The highly substituted Sepharose derivatives retained very good flow properties.

Figure 5 illustrates the effect of pH and ionic strength on the chromatographic patterns. Although stronger binding appears to occur with buffer of lower ionic strength (0.01 M), this should not be used since some proteins, such as pancreatic ribonuclease, will adsorb nonspecifically to unsubstituted or inhibitor-coupled Sepharose under those conditions. Figure 5 shows the patterns obtained with a number of other enzymes, emphasizing the specificity of the α-chymotrypsin-specific Sepharose columns. Very small chymotryptic impurities

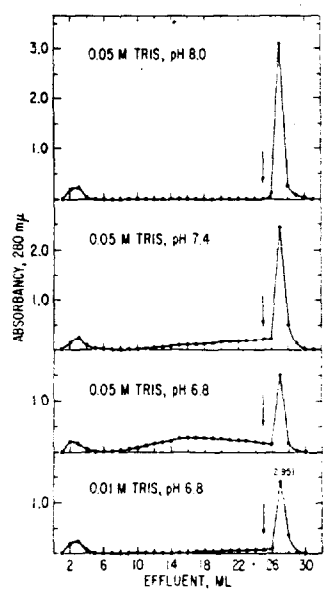

FIG. 5.—Effects of pH and ionic strength on affinity adsorption of α-chymotrypsin on a column (0.5 × 5 cm) of Sepharose coupled with ϵ-amino caproyl-D-tryptophan methyl ester. A sample containing 2.5 mg of α-chymotrypsin in 0.5 ml of buffer was applied to the column. Elution of α-chymotrypsin was performed with 0.1 M acetic acid, pH 3.0 (*arrow*). Other conditions were as in Fig. 2. The first peak (tubes 2–4) was devoid of chymotrypsin activity,[18] and the specific activity of the subsequently eluted protein was constant.

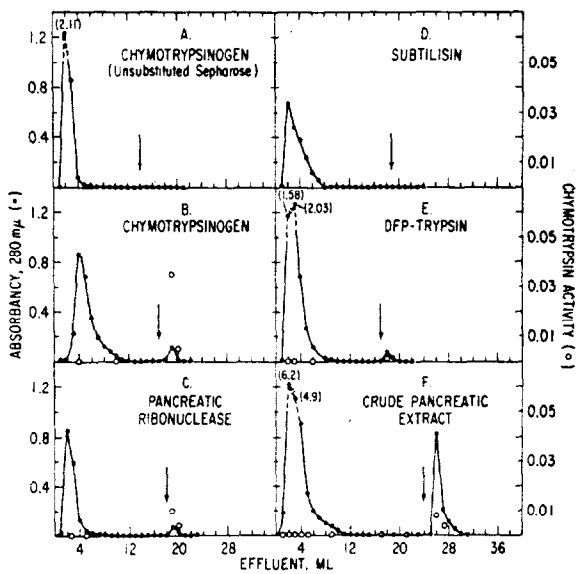

FIG. 6.—Chromatographic patterns obtained by passing several enzyme preparations through a column (0.5 × 5 cm) of Sepharose coupled with ϵ-amino caproyl-D-tryptophan methyl ester. The column was equilibrated and run with 0.05 M Tris-Cl buffer, pH 8.0. Approximately 3 mg of protein (A through E), dissolved in 0.5 ml of the same buffer, was applied to each column. Chymotrypsin was eluted with 0.1 M acetic acid (*arrow*). The sample used in F consisted of 1 ml of a supernatant (280 mμ absorbancy, about 15) obtained by dissolving about 100 mg of an acetone powder of bovine pancrease in 3 ml of Tris-Cl, pH 8.0, followed by centrifugation for 20 min at 4000 × g. Enzyme activity is expressed as the change in absorbancy (at 256 mμ) per minute per 5 μl of sample, with benzoyl-L-tyrosine ethyl ester as substrate.[18] Other conditions were as in Figs. 4 and 5.

can be readily removed from other enzymes by these techniques. This may be a useful way of removing chymotryptic impurities from other proteases used for structural studies, where even small traces can lead to unexpected cleavages.

It is of interest that chymotrypsinogen A is very slightly but significantly retarded by the chymotrypsin-specific column (Figs. 6*A* and *B*), suggesting that this precursor is capable of weakly recognizing this substrate analog. A proteolytic enzyme of broad substrate specificity, subtilisin, is not adsorbed (Fig. 6*D*). A small amount of chymotryptic-like protein could be readily separated from a crude pancreatic digest (Fig. 6*F*). This material contained all of the chymotryptic activity[18] of the crude digest, but the exact nature of this material or the reason for the low specific activity were not determined.

Unlike the results obtained with the nuclease-specific Sepharose system (Fig. 3), the chymotrypsin-specific Sepharose derivative was relatively ineffective in stopping the hydrolysis of benzoyl-L-tyrosine ethyl ester when added to a reaction mixture. Attempts to elute α-chymotrypsin from a column, like that shown in Figure 4, with a 0.01 *M* solution of the above substrate were unsuccessful. However, elution did occur with a 0.018 *M* solution of β-phenylpropionamide, an inhibitor with a K_i of 7 mM.[19] If the Sepharose column was equilibrated and developed with 0.0058 *M* β-phenylpropionamide solution, α-chymotrypsin was only moderately retarded. These results indicate, again, that the processes involved in the separations are clearly related to the functional affinity of the enzymatic binding site for specific structural substances.

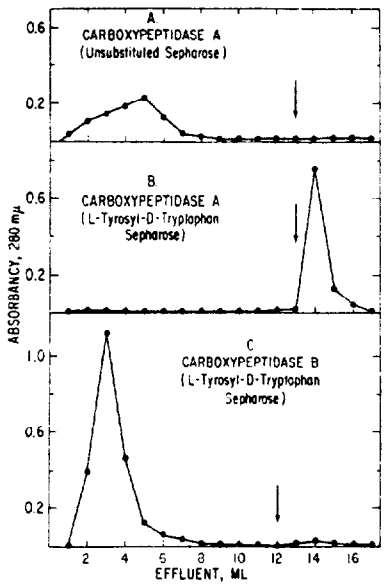

FIG. 7.—Affinity chromatography of carboxypeptidase A on a column (0.5 × 6 cm) of Sepharose coupled with L-tyrosine-D-tryptophan. The buffer used was 0.05 *M* Tris-Cl, pH 8.0, containing 0.3 *N* NaCl. About 1 mg of pure carboxypeptidase A (*A*, *B*) and 1.8 mg of carboxypeptidse B (*C*), in 1 ml of the same buffer, were applied to the columns. Elution was accomplished with 0.1 *M* acetic acid (*arrow*).

Carboxypeptidase A: A specific adsorbent for this enzyme was prepared by coupling the dipeptide, L-tyrosine-D-tryptophan, to Sepharose. In the coupling procedure, about 60 μmoles of the dipeptide inhibitor were added per milliliter of Sepharose, and approximately 8 μmoles per ml of Sepharose were coupled. Figure 7 illustrates that this enzyme was strongly adsorbed by a column containing such a substituted Sepharose. The yields obtained upon elution were again quantitative.

Discussion.—In recent years there has been considerable interest in the covalent attachment of biologically active compounds (i.e., enzymes, antibodies, and antigens) to insoluble polymers.[20] These materials, especially the derivatives of cellulose, have found use in the purification of antibodies,[21]

nucleotides,[22] complementary strands of nucleic acids,[23] certain species of transfer RNA,[24] and enzymes.[1-3]

The principles and procedures, as illustrated and outlined in this communication, should be of value in the purification and isolation of a great many biologically active proteins or polypeptides which can reversibly and specifically bind to small molecules. If the latter are not chemically altered during the reversible adsorption process (e.g., an inhibitor), and if an amino group can be introduced in a region of its structure in such a way that binding to the macromolecule is unaffected, the procedures outlined here should be directly applicable.

Summary.—Principles and techniques for selective enzyme purification by affinity adsorption to inhibitor-Sepharose columns are presented and illustrated by experiments performed on staphylococcal nuclease, α-chymotrypsin, and carboxypeptidase A. Inhibitory substrate analogs linked to Sepharose provide adsorbents on which enzymes can be purified rapidly and completely in a single step.

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